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Development of transgenic alfalfa plants containing the foot and mouth disease virus structural polyprotein gene P1 and its utilization as an experimental immunogen

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Abstract

The use of transgenic plants as vectors for the expression of viral and bacterial antigens has been increasingly tested as an alternative methodology for the production of experimental vaccines. Here, we report the production of transgenic alfalfa plants containing the genes encoding the polyprotein P1 and the protease 3C of foot and mouth disease virus (FMDV). The immunogenicity of the expressed products was tested using a mouse experimental model. Parenterally immunized mice developed a strong antibody response and were completely protected when challenged with the virulent virus. This report demonstrates the possibility of using transgenic plants to express polyprotein P1 and the protease 3C of FMDV and their utilization as effective experimental immunogens.

Keywords: Foot and mouth disease virus; Transgenic plants; Experimental vaccines

1. Introduction

The use of transgenic plants for the expression of recombinant antigens has been increasingly used for the production of experimental immunogens [1–10] since they are, potentially, an inexpensive source of antigens to be parenterally administrated or, more interestingly, to be used as edible vaccines [1–3,10]. Although a very promising technology, most of the antigens expressed to date have been epitopes or single proteins containing linear epitopes [8,10] or adopting simple tridimensional structures [3,4,9]). However, the practical use of transgenic plants as a source of antigen for vaccine production would undoubtedly require, in most cases, the expression of more complex antigenic structures.

Foot and mouth disease virus (FMDV) is the causative agent of a very significant economic disease affecting meat and milk producing domestic animals [11]. Current vaccines are based on the utilization of inactivated virus and, although they have proved to be effective tools for the prevention of the disease, their production is both costly and risky because of manipulation of massive amounts of virulent virus could result in virus dissemination [11]. Thus, it is important to develop new approaches, which could provide practical alternatives to the current methodology of vaccine production.

With that scope, we have focused on the development of alternative methods of FMDV vaccine production using transgenic plants as bioreactors. We have successfully demonstrated that the FMDV VP1 protein, which carries critical epitopes responsible for the induction of protective neutralizing

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antibodies, could be expressed in plants of *Arabidopsis thaliana*, alfalfa and potato [12–14], and was able to elicit a virus-specific antibody response and protection against the virulent challenge after parenteral or oral administration.

Here, we report the production of transgenic alfalfa plants expressing the FMDV VP1 polyprotein and show that the expressed products are immunogenic in a mouse experimental model by inducing a strong FMDV-specific antibody response against complete virus particles and viral subunits as well as a complete protection against the experimental challenge with the virulent virus. These results demonstrate, for the first time, the possibility of using transgenic plants to express complex antigenic structures and their utilization as effective experimental immunogens.

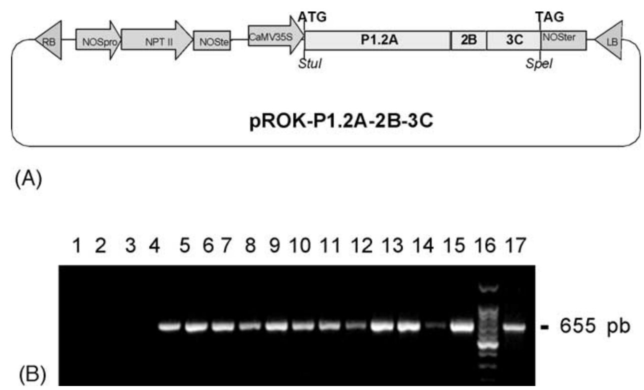

Fig. 1. (A) Schematic representation of pRok-P1-3C. (B) Detection of the FMDV genes in pRok2P1-3C transgenic plants using PCR. Lane description: non-template (1), DNA from non-transformed plants (2), DNA from a plant transformed with a non-related gene (3), DNA from pRok2P1-3C transformed plants (4 through 15), MW marker (16) and pRokP1-3C plasmid as positive control (17).

2. Materials and methods

2.1. Production of transgenic plants of alfalfa

A 3327 bp DNA fragment (spanning the P1-3C protein coding region) was introduced in the genome of transgenic plants. P1-3C encodes for the FMDV O1C genomic regions P1 (1A, 1B, 1C, 1D), 2A, the first 61 amino acid residues of the N terminus of 2B, the complete sequence of 3B1, 3B2, 3B3, 3C and the first 16 amino acid residues of the N terminus of 3D. The construction also contains a start and a stop codon at positions 1 of 1A and following the 16th codon of 3D, respectively.

P1-3C was obtained by sequential cloning of different individual fragments which were previously amplified by RT-PCR from viral RNA intp plasmid pUC19 (Biolabs). The vector obtained was named pUC-P1.2A-2B-3C. The primers used, the region of the FMDV genome that the span and the restriction sites added for cloning are summarized in Table 1.

The complete P1-3C fragment was then removed from pUCP1.2A-2B-3C by digesting with *Stu*I/*Spe*I and cloned into the *Stu*I/*Spe*I sites in the polylinker of the binary pRok plasmid [13] obtaining the recombinant plasmid pRokP1-3C which was used in the plant transformation protocols. In pRokP1-3C, the P1-3C coding region is placed under the control of the cauliflower mosaic virus (CaMV 35S) transcriptional promoter (Fig. 1A).

Transformation of alfalfa was performed, using *Agrobacterium tumefaciens*-mediated transformation, exactly as described in Wigdorovitz and co-workers [12].

2.2. PCR and RT-PCR analyses

The presence of the recombinant genes in transgenic alfalfa plants was detected in total nucleic acids samples extracted from, approximately, 50 mg of leave tissues following the protocol described elsewhere [12,13]. The P1 gene was specifically amplified using a pair of primers which span from position 63 of VP1 to position 27 of 2A (forward primer: 5′ AGCGGATCCTGTCATGGCCACTGTTGAA 3′; reverse primer: 5′ AAGGGGATCCTCTAGAGTCTACTTGAG 3′), amplifying a DNA fragment of 655 bp.

The transcription of the transgenes was analyzed by performing RT-PCR specific for each of the transcripts in the plant extracts. Total RNA extraction was performed from 1 g of fresh leaves following the protocol described elsewhere [12]. PCR was performed using the same primers described above for detecting the presence of the transgenic gene in the plants. Southern blot analysis of the P1-3C amplified fragment was performed by using as probe a PCR DNA fragment amplified between positions 2898 and 3542, which was α-^{32}P labeled by random priming using a commercial kit (Promega).

2.3. Detection of the presence of FMDV empty capsids in the transgenic plants

Freshly harvested leaves from either pRokP1-3C or non-transformed plants were fixed in 2% glutaraldehyde, post-

Table 1
Primers used for the construction of P1.2A-2B-3C

Primer	Sequence (5′ to 3′)	FMDV genome stretch
P1.2A	Forward: TGAATTC$_{EcoRI}$AAAGGCCT$_{StuI}$ATGGGGGCTGCACAATCC	1317–3575
	Reverse: GACTCTAGAGGGCCC$_{ApaI}$AGGTTGGACTC$_{XbaI}$GAC	
2B	Forward: TCCAACCCTGGGCCC$_{ApaI}$TCTTTTTTC	3570–3755
	Reverse: GATTCTAGA$_{XbaI}$GTACCAGGGTTTGGC	
3C	Forward: GAATCTAGA$_{XbaI}$TACGCCGACCACTC	5454–6347
	Reverse: TGCAAGCTTACTAGT$_{HindIII}$ $_{SpeI}$CTACATCACGTGAACGCGCTC	

fixed with 2% osmium tetraoxide, dehydrated, and stained with the uranilo/Reinold's method. Samples were observed in an EM at 50,000× magnification (Departamento de Microscopía electrónica, Facultad de Farmacia y Bioquimica. Universidad de Buenos Aires).

2.4. Analysis of antibody response to plant-expressed FMDV polypeptides

Adult (60–90-day-old) male BALB/c mice were immunized intraperitoneally (i.p.) on days 0, 15, 30 and 45 with either leaf extracts from transgenic plants expressing P1-3C or leaf extracts from transgenic plants expressing an unrelated foreign gene (150 μl of leaf extract, containing 15–20 mg of fresh leaf tissue, in incomplete Freund's adjuvant per animal per injection). Mouse sera were evaluated for the presence of anti FMDV specific antibodies by ELISA and Western blot exactly as described in Wigdorovitz and co-workers [12–15].

Neutralizing antibody response and challenge experiments were performed as described in Dus Santos et al. [16].

3. Results

3.1. Production and genetic analysis of transformed plants

Nine lines of transgenic plants containing the P1-3C gene were initially produced. The presence of the P1-3C genes was detected in all the pRokP1-3C transformed plants by amplification of a PCR product of the expected size (655 bp) that was regularly absent in the non-transformed plants (Fig. 1B). Additionally, the complete sequence of 3C was detected in the transgenic plants (data not shown).

RT-PCR of pRokP1-3C transformed plants consistently resulted in products corresponding to the recombinant P1-3C genes (Fig. 2A). Furthermore, the specificity of the P1-3C amplified product obtained by RT-PCR was confirmed by Southern blot (Fig. 2B). The probe only hybridized with PCR products from P1-3C transgenic plants; no reactivity was observed in non-transformed plant or a plants transformed with a non-related gene.

3.2. Induction of immune response in mice intraperitoneally immunized with foliar extracts

Balb/c mice were immunized i.p. at days 0, 15, 30 and 45 with approximately 15–20 mg of plant tissue emulsified in Incomplete Freund's Adjuvant (plant extracts were prepared by macerating approximately 150 mg of frozen leaves, containing 0.005–0.01% total soluble protein, in 1 ml of PBS-T). Ten days after the last inoculation, animals were bled and the sera analyzed for the presence of anti-FMDV antibodies. Antibodies raised in the immunized mice presented a specific response in ELISA against the structural protein VP1, as demonstrated by their reactivity to the synthetic peptide p135-160, and to purified FMDV particles (Fig. 3A).

Based on these results, plant A was chosen to further continue with the immunological analysis of the product expressed in alfalfa tissues. A new group of mice was immunized with the vaccine formulated from this plant and the antibody response was analyzed by ELISA (Fig. 3B). The specificity in the antibody response was confirmed by Western blot, using purified FMDV as antigen. Different pools of sera from the immunized mice specifically recognized the FMDV structural proteins with a similar pattern of reactivity as a pool of sera from mice experimentally infected with FMDV (Fig. 3C).

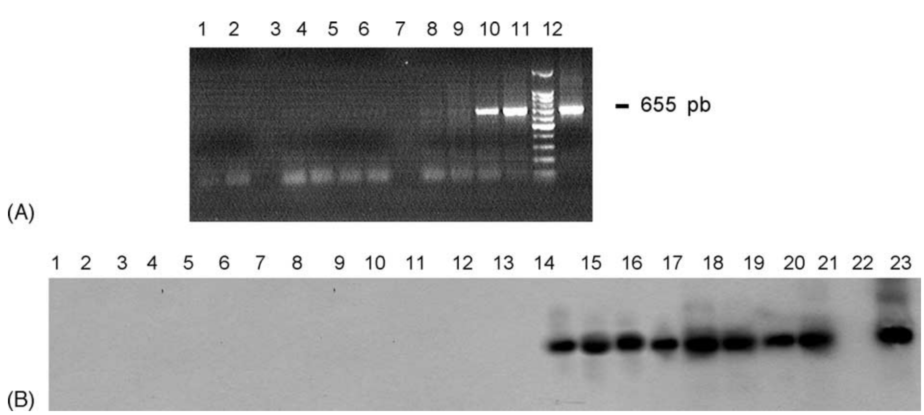

Fig. 2. Detection of transcription of the VP1 gene in pRokP1-3C transgenic plants. (A) RT-PCR. Lane description: RNA samples shown in lanes 1, 3–6 were subjected to PCR amplification without the previous RT reaction as control for DNA contamination. RNA samples shown in lanes 2, 7–10 were subjected to PCR amplification after RT reaction. Non-template (1 and 2), RNA from non-transformed plants (3 and 7), RNA from pRok2P1-3C transformed plants (5/9 and 6/10), MW marker (11), DNA for the control of amplification (12). (B) Southern blot analysis of the FMDV RT-PCR products. Lane description: RNA samples shown in lanes 1, 3–12 were subjected to PCR amplification without the previous RT reaction while RNA samples shown in lanes 2, 15–22 were subjected to PCR amplification after RT reaction. Non-template (1 and 2), RNA from non-transformed plants (3 and 13), RNA from a plant transformed with a non-related gene (4, 14), RNA from pRok2P1-3C transformed plants (5–12 and 15–22), and DNA for the control of amplification (23).

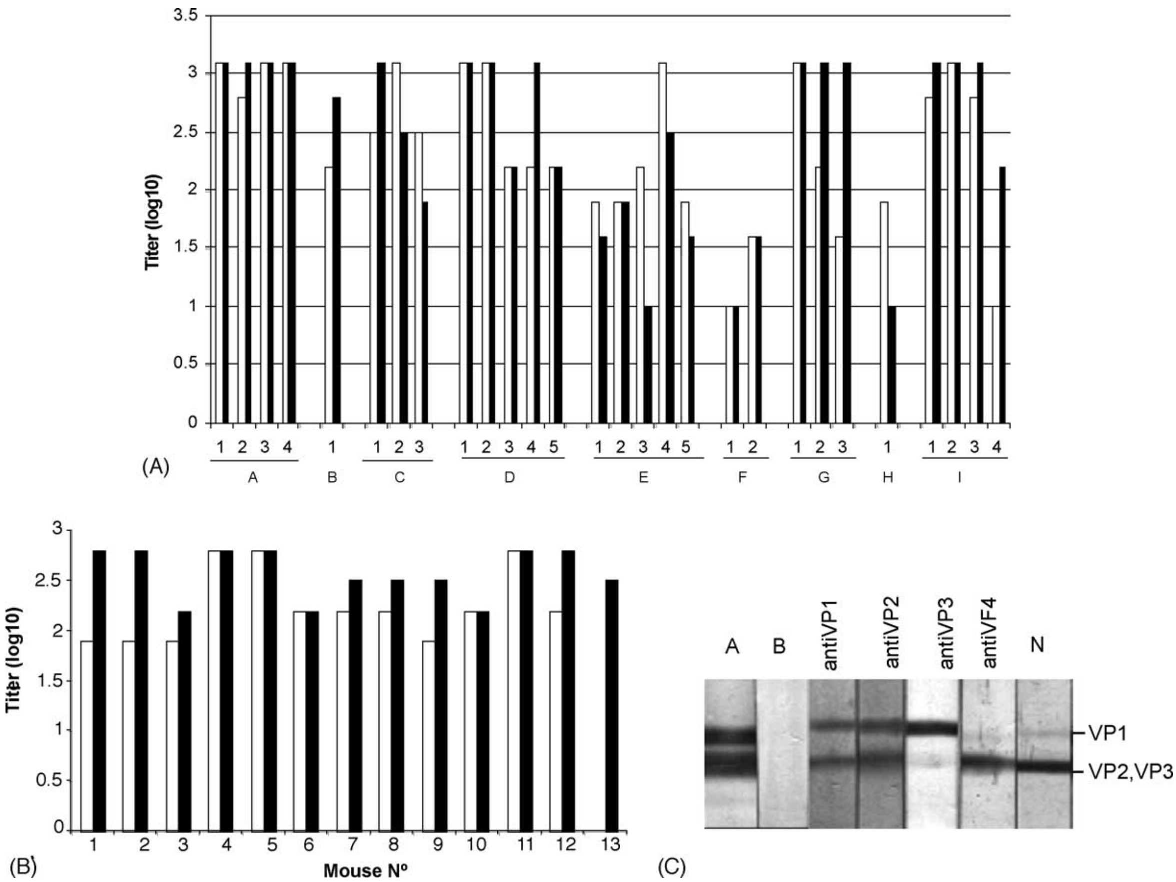

Fig. 3. Detection of antibodies to p135-160 (p1) (empty bars) and anti-FMDV particles (FMDV) (filled bars) in mice intraperitoneally immunized with leaves extracts from pRok2P1-3C using ELISA. Serum titers are expressed as the $\log_{10}$ of the reciprocal of the highest serum dilution which present OD readings above of the mean OD readings + 3S.D. of sera from 5 animals immunized with plants transformed with a non-related recombinant gene. (A) Lower case letters in the bottom of the bars correspond to different mice immunized with each of the different plant lines (1 through 9). (B) Numbers at the bottom of the bars indicate different mice immunized with plant A. (C) Anti-FMDV antibodies detected by Western blot in the sera from mouse immunized with leaf extracts from pRok2P1-3C transformed plants. Lane description: different pools of sera from mice immunized with pRok2P1-3C transformed plants (A and B) or from plants transformed with a non-related recombinant gene (N). As control, sera from mice specifically reacting with each of the FMDV structural proteins are included.

Furthermore, mice immunized with the plant A extract developed a strong neutralizing antibody response (Table 2).

3.3. Protection of the immunized mice against the virus challenge

To test the effectiveness of the induced immune response in preventing virus infection, the vaccinated mice were experimentally challenged with infectious FMDV. Mice were inoculated i.p. with 10^4 $SM_{50}LD$ of FMDV O1C and the absence of viremia 36 h later was considered as an indicator of protection [13,15]. All immunized animals were protected against the experimental infection, indicating the capability of the plant-expressed FMDV polyprotein P1 to induce a protective antibody response (Table 3).

Table 2

Mouse N°	SNI
1[a]	2.2
2[a]	2.3
3[a]	2.1
4[a]	2.1
5[b]	0.6
6[b]	0.6

[a] Mice i.p. immunized with plant A extract.
[b] Mice i.p. immunized with a plant transformed with a non-related gene.

Table 3
Protection against FMDV challenge in mice i.p. immunized with different plant extracts

Mice immunized i.p. with	Protection rate[a]
pRok2P1-3C (plant A)	20/20 (100%)
pRok2P1-3C (pool other plants)	21/23 (91%)
Non-transformed plant extracts	0/10 (0%)
Mock immunized	0/6 (0%)

[a] Protection is expressed as a Number of protected mice/Number of challenged mice.

4. Discussion

The use of transgenic plants for antigen production has received further experimental support from several groups who have reported the expression of different antigens using this methodology [1–10]. Nevertheless, most antigens expressed in transgenic plants constitute unique proteins or simple structures. Hepatitis B surface antigen [3], Norwalk virus capsid protein [4] and human papilloma virus capsid protein [9] are the only antigenic structures with some degree of complexity that have been stably expressed in plants. The practical use of transgenic plants as a source of antigen would be supported by the possibility of producing complex antigenic structures. The aim of this work was, thus, to express FMDV polyprotein and protease 3C in transgenic alfalfa plants. Production of FMDV empty capsid require proteolytic precessing of P1 by protease 3C to generate structural proteins, which assemble themselves to form the viral capsid.

Traditional, inactivated vaccines against FMDV have proven to be effective tools for the prevention of the disease. Although efficacious, their production is both costly and risky because of manipulation of massive amounts of virulent virus could result in virus dissemination [11]. Additionally, the immune response to the vaccine interferes with the ability to detect vaccinated animals that have subsequently become infected and could carry and shed the virus, creating an obstacle to re-instating disease-free status to countries/regions that vaccinate to control outbreaks. Many diagnostic tests are based on the detection of antibodies to viral non-structural proteins that are present in low concentration in traditional FMDV vaccines and are poorly immunogenic in vaccine preparations. Expression of empty capsid of the virus constitutes a feasible strategy to circumvent these disadvantages [17].

The presence of the transgenes in the plant genome and their specific transcriptional acitivity were detected in all transgenic plants produced. Expression of P1 polyprotein in transgenic alfalfa plants induced, in an experimental murine model, a strong neutralizing antibody response. Additionally, immunized animals were completely protected against the challenge with virulent virus. These results indicate that the expressed products contain conserved protective epitopes.

Although no definitive data have been produced, electron microscopy analysis showed the presence of virus-like spherical structures of 30 nm size in the transgenic plant expressing P13C. These structures were absent in a plant carrying a non-related gene (data not shown). The identity of these structures will be confirmed by immunomicroscopy.

Previous data obtained by our group expressing VP1 protein as well as the results presented in this work showed that the levels of antigen expression are still low for practical purposes. In order to circumvent this limitation, we are working to optimize product yield by utilizing alternative promotors to direct foreign protein expression.

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